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Aging promotes a different phosphatidic acid utilization in cytosolic and microsomal fractions from brain and liver

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Abstract

Among the morphological and biochemical changes taking place in the membranes of aged tissues, we reported in previous studies on alterations in phospholipid synthesis and phospholipid-specific fatty acid composition. Phosphatidic acid (PA) and diacylglycerol (DAG) are central intermediates in phosphoglyceride and neutral lipid biosynthetic pathways and have also recently been implicated in signal transduction. The present paper shows the effect of aging on phosphatidate phosphohydrolase (PAPase) activiy, which operates on phosphatidic acid to synthesize diacylglycerol. Two forms of mammalian PAPase can be indentified on the basis of subcellular localization and enzyme properties, one involved predominantly in lipid synthesis (PAP 1) and the other in signal transduction (PAP 2). Microsomal and cytosolic fractions of brain and liver from 3.5-month-old (adult) and 28.5-month-old (aged) rats were used. PAPase isoform activities were differentiated on the basis of N-ethylmaleimide (NEM) sensitivity and Mg^{2+}-dependency. Our results demonstrate that aging caused PAP 2 to increase in brain microsomal fractions but did not affect PAP 1, whereas in brain cytosolic fractions, it caused a strong decrease in PAP 1 (57%). The distribution of enzymes between microsomes and cytosol changed in aged rats with respect to adult rats, showing a translocation of PAP 1 from cytosol to microsomes. In addition, an increase in the production of monoacylglycerol (MAG) was observed in microsomes from aged brain. PAP 2 activity in liver microsomal fractions from aged rats showed no changes with respect to adult rats whereas PAP 1

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Abbreviations: CYT, cytosolic fraction; DAG, diacylglycerol; DAGL, diacylglycerol lipase; MAG, monoacylglycerol; DTT, dithiotreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethylether) N,N,N′N′-tetraacetic acid; MIC, microsomal fraction; NEM, N-ethylmaleimide; PAPase, phosphatidate phosphohydrolase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin layer chromatography; TRIS, tris[hydroxymethyl]aminomethane

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activity increased 228% in microsomal fractions and 76% in cytosolic fractions in this tissue. The distribution of PAP 1 activity between microsomal and cytosolic fractions in liver tissue was also affected in aged rats, indicating a translocation of this form of the enzyme from cytosolic to microsomal fractions. The production of monoacylglycerol in liver microsomes also increased, whereas there was a decrease in MAG formation from cytosolic fraction. The changes observed in the two PAPase forms in brain and liver of aged rats with respect to adult rats suggest that PA is differently utilized by the PAPase isoforms, probably generating aging-related DAGs different to those present in adults and required for specific cellular functions. The changes observed in liver PAP 1 from aged with respect to adult rats suggest that such changes could be related with modifications in lipid homeostasis induced by age-altered hormonal balance. However, PA-modified utilization during aging through PAP 2 activity could be related to alterations in neural signal transduction mechanisms.

Keywords: Aging; Brain; Diacylglycerol; Liver; Phosphatidate phosphohydrolase; Phosphatidic acid

1. Introduction

Aging is a complex process involving both morphological and biochemical changes. The molecular basis for the numerous physiological changes that occur with aging are not well understood. One mechanism that could contribute to these changes is the alteration in cell membrane composition and its biophysical properties. These latter have been shown to affect a wide variety of processes, including ion transport, signal recognition and transduction, and the regulation of enzymatic activities (Bruce et al., 1985). Aging may have some effect on biochemical parameters related to phospholipid metabolism in the nervous system. One of the most important functions of membrane lipids is to maintain physiological properties, mainly membrane fluidity. Thus, any effect on phospholipid metabolism may provoke membrane function alterations. One of the major factors known to modify membrane structure and physical characteristics is related to compositional changes such as cholesterol content (Yeagle, 1985) and the degree of saturation and unsaturation of the esterified acyl groups (Dobretsov et al., 1977; Stubbs and Smith, 1984).

Phosphatidate (PA) lies at a branch-point in glycerolipid synthesis, which is thought to be under physiological and pharmacological control (Butterwith et al., 1984; Sturton and Brindley, 1980). PAPase is a key enzyme of glycerolipid metabolism, controlling PA conversion into diacylglycerol (DAG) (Jamal et al., 1991) for the synthesis of triacylglycerols (TAG), phosphatidylcholine (PC) and phospatidylethanolamine (PE). A differential effect of aging on the biosynthesis of ethanolamine and choline phosphoglycerides in brain and liver has been reported (Brunetti et al., 1979; Gaiti et al., 1979). Phospholipid base-exchange activity of liver microsomes has been shown to decrease with age, manifesting no changes in brain microsomes (Gaiti et al., 1979).

Studies in our laboratory have shown that the zwitterionic glycerophospholipid biosynthesis measured through the incorporation of [2-^{3}H]-glycerol seems not to be affected in brain tissue suspension of 28.5-month-old rats (Ilincheta de Boschero et al., 2000a). Research on changes in PAPase activity in rat liver from birth to adult age has been carried out (Cascales et al., 1988; Goldberg et al., 1983; Savolainen et al., 1981),

though relatively few studies deal with aging-related changes in the activity of specific lipid metabolism enzymes in brain and liver.

Our interest focuses mainly on the effect of aging on the utilization of PA by PAPase in brain and liver tissues. *It has been useful to compare the results of PAPase activity obtained both in brain and liver because the latter exhibits a more homogenous and less complex structure as compared to that of brain in which interactions among cells of different origin and function are very complex*. Two types of mammalian PAPase hydrolyse PA to yield DAG: the cytosolic/microsomal Mg^{2+}-dependent enzyme (PAP 1) is selectively inactivated by the thioreactive N-ethylmaleimide (NEM) (Martin et al., 1991) and has not been purified, nor has its cDNA been cloned (Vance, 1998). The Mg^{2+}-independent, NEM-insensitive enzyme (PAP 2) (Martin et al., 1991) is located in the plasma membrane; it exists in several isoforms, has been purified, its cDNA has been cloned (Hooks et al., 1998; Roberts et al., 1998) and it is present in many cell types (Billah and Arthes, 1990; Jamal et al., 1991). In the present paper, the hydrolisis of phosphatidic acid by both enzymes was studied in brain and liver of adult and aged rats. Labeled DAG, as a product of different PAPase activities, was also metabolized to monoacylglycerol. Significant and different modifications in the metabolism of PA by PAP 1 and PAP 2 activity were observed during aging in both tissues.

2. Materials and methods

2.1. Animals and tissue dissection

Male Wistar rats (3.5- and 28.5-months old, three animals per group in each of two different experiments) were killed by decapitation. After decapitation, cerebral hemispheres and liver were dissected rapidly and homogenized in a medium containing 0.32 M sucrose, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4) to a final concentration of 30% (w/v).

2.2. Preparation of subcellular fractions

Homogenates were centrifuged at 11500 × g for 20 min and washed once to produce a nuclear-mitochondrial pellet. The post-mitochondrial supernatant was centrifuged at 130,000 × g for 45 min to obtain the microsomal (MIC) and the cytosolic (CYT) fraction.

Marker enzymes for plasma membrane and endoplasmic reticulum were measured. 5′ Nucleotidase and NADPH cytochrome C reductase (Hodges and Leonard, 1974) were assayed in the homogenate and subcellular fractions (nuclear plus mitochondrial fraction, microsomal and cytosolic fractions).

2.3. Determination of PAPase activity and MAG production

2.3.1. PAPase actvity

PAPase activities were differentiated on the basis of Mg^{2+}-dependency and NEM-sensitivity (Jamal et al., 1991; Martin et al., 1991). PAPase Mg^{2+}-dependent activity was determined in a medium containing 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 3 mM

Mg^{2+} and 200 μg of microsomal or cytosolic protein. The reaction was started by adding 0.6 mM [^{3}H]phosphatidic acid plus 0.4 mM dipalmitoyl phosphatidylcholine prepared by sonication as described below. The assay for the Mg^{2+}-independent form of PAPase was carried out in the presence of 1 mM EDTA plus 1mM EGTA. The difference between PAPase activity in the presence of Mg^{2+} and PAPase activity in the presence of 1 mM EDTA plus 1 mM EGTA was considered as Mg^{2+}-dependent activity.

For the determination of PAPase activity by the assay of NEM sensitivity the following determination was carried out: PAP 2 activity was determined by assaying the enzyme in 50 mM Tris-maleate buffer, pH 6.5, containing 1 mM DTT, 1 mM EDTA plus 1 mM EGTA and 4.2 mM NEM, in a final volume of 0.265 ml. The reaction was started by adding 0.6 mM of [^{3}H] phosphatidate to 200 μg of microsomal or cytosolic protein.

PAP 1 activity was determined in an assay containing 50 mM Tris-maleate buffer, pH 6.5, 1 mM DTT, 3 mM Mg^{2+} and 200 μg of microsomal or cytosolic protein, in a final volume of 0.265 ml. The reaction was started by adding 0.6 mM of [^{3}H]phosphatidate plus 0.4 mM phosphatidylcholine. Parallel incubations were carried out after pre-incubating the enzyme with 4.2 mM NEM for 10 min. The difference between these two activities was considered as PAP 1. Assays for the determination of PAPase were conducted at 37°C for 40 min. The enzyme assay was stopped by adding chloroform/methanol (2:1, by vol.). Blanks were prepared identically, except that membranes were boiled for 5 min before being used. 1,2-Diacyl[^{3}H]glycerol and [^{3}H]monoacylglycerol, PAPase activity products, were isolated and measured as described below. PAPase activity was expressed as the sum of nmol of ([^{3}H]diacylglycerol and [^{3}H]monoacylglycerol) × (hour) $-^1$ × $(\text{mgprot})^{-1}$ (Pasquaré de García and Giusto, 1986).

MAG synthesis in cytosolic and membrane fractions from both tissues was measured by monitoring the release of [2-^{3}H] monoacylglycerol from 1,2 diacyl-[2-^{3}H] glycerol (as a PAPase product from NEM S or PAP 1 activity) (Pasquaré and Giusto, 1993). MAG synthesis was expressed as dpm of [2-^{3}H] monoacylglycerol × $(\text{hour})^{-1}$ × $(\text{mgprot})^{-1}$. Water soluble products such as lysophosphatidic acid and glycerol phosphate was also monitored.

2.4. Preparation of radioactive 1,2-diacyl-sn-glycerol-3-phosphate

Radioactive phosphatidic acid was obtained from [^{3}H]phosphatidylcholine which had been synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as previously described (Pasquaré de García and Giusto, 1986). Lipids were extracted from the tissue as described by Folch et al., 1957. [^{3}H]Phosphatidylcholine was isolated by TLC and eluted therefrom (Arvidson, 1968). [^{3}H]PC was then hydrolyzed with phospholipase D (Kates and Sastry, 1969) and [^{3}H]phosphatidate, the hydrolysis product, was purified by one-dimensional TLC on silica gel H developed with chloroform/methanol/acetic acid/acetone/water (9:3:3:12:1.5, by vol.). The substrate was eluted from silica gel with neutral solvents to avoid lysophosphatidic acid formation and then converted into free acid by washing it twice according to Folch et al., 1957, using an upper phase containing 0.1 M sulfuric acid and then an upper phase containing water. Radioactivity and phosphoros content (Rouser et al., 1970) were measured to determine specific radioactivity. [^{3}H]PA had a specific radioactivity of 0.1–0.2 μCi/μmol.

When PAPase activity was determined for Mg^{2+}-dependency, the substrate was prepared as a dispersion into the same buffer as for incubation and sonicated for 5 min. To determine PAP 1 activity, the substrate was prepared by sonicating 3.33 mM [^{3}H]-phosphatidate (0.1–0.2 μCi/μmol) and 2.22 mM dipalmitoyl phosphatidylcholine in 5.56 mM EGTA and 5.56 mM EDTA. For the determination of PAP 2 activity, an identical emulsion was prepared as indicated above except that phosphatidylcholine was omitted (Jamal et al., 1991; Martin et al., 1991).

2.5. Extraction and isolation of lipids

Lipids were extracted with chloroform/methanol (2:1, by vol.), and extracts were washed with 0.2 volumes of $CaCl_2$ (0.05%) (Folch et al., 1957). The presence of soluble products was monitored through radioactivity measurements of the water phase. Neutral lipids were separated by gradient-thickness thin-layer chromatography on silica gel G (Giusto and Bazán, 1979) and developed with hexane/diethyl ether/acetic acid (35:65:1, by vol.). Once the chromatogram was developed, [^{3}H]PA and phospholipids were retained at the spotting site. Lipids, DAG and MAG were visualized by exposure of the chromatograms to iodine vapours, and scraped off for counting by liquid scintillation after the addition of 0.4 ml water and 10 ml 5% omnifluor in toluene/Triton X-100 (4:1, by vol.).

2.6. Other methods

Protein and lipid phosphorus were determined according to Bradford, 1976; Rouser et al., 1970, respectively.

2.7. Statistical analysis

Statistical analysis was performed using Student's *t*-test, with values representing the mean ± S.D. of the total number of samples indicated in each legend. In the case of the percent ratios, (Fig. 2) S.D. (standard deviation) was calculated according to the ad-hoc statistical treatment (Johnson and Kotz, 1969).

3. Results

We distinguished between Mg^{2+}-dependent (PAP 1) and Mg^{2+}-independent (PAP 2) activities by using assays containing either Mg^{2+} or EDTA plus EGTA, respectively (Ide and Nakazawa, 1985). This technique works relatively well once bivalent cations have been removed from the phosphatidate substrate. In the present study, bivalent cations were removed by treating phosphatidate with EDTA and EGTA during the substrate preparation.

A further approach used was to selectively measure PAPase activity in tissues by means of NEM, which inhibits Mg^{2+}-dependent PAPase (PAP 1) activity but does not affect Mg^{2+}-independent activity (PAP 2) (Jamal et al., 1991).

Fig. 1 shows the results using NEM to selectively measure NEM-insensitive (PAP 2) and NEM-sensitive (PAP 1) *in microsomal and cytosolic fractions of brain homogenates from adult and aged rats*. According to our results, NEM-I (PAP 2) activity was only

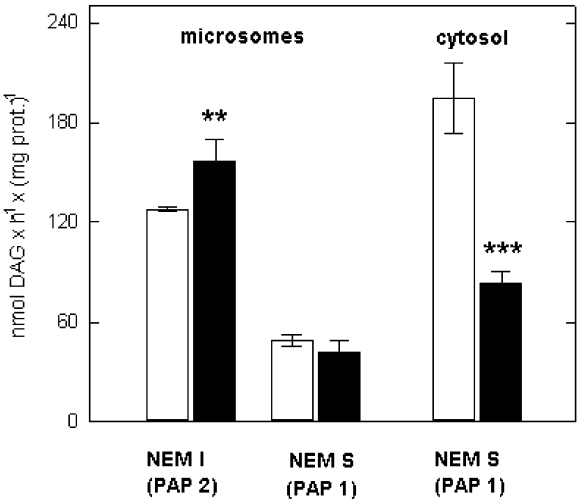

Fig. 1. Metabolization of phosphatidic acid by NEM-S (PAP 1) and NEM-I (PAP 2) phosphatidate phosphohydrolase activities in microsomal and cytosolic fractions of brain homogenates from adult and aged rats. PAP 1 activity was determined from the total activity measured with Mg^{2+} (3 mM) and NEM (4.2 mM). Parallel incubations were carried out after pre-incubating the enzyme with 4.2 mM NEM for 10 min. The difference between these two activities was taken as PAP 1; adults (□) and aged (■). PAP 2 represented PAPase activity measured in the presence of NEM (4.2 mM), without Mg^{2+} (1mM EDTA plus 1 mM EGTA were carried out with the substrate); adults (□) and aged (■). Enzymatic activities were expressed as nmol of DAG released × $hour^{-1}$ × (mg prot.)$^{-1}$. Each value is the mean ± SD of three individual samples from different animals (three adults and three aged rats). (**$p < 0.01$; ***$p < 0.001$).

observed in brain microsomal fractions, where it was 2.4 times higher than the activity recorded for the NEM-S (PAP 1) form (Fig. 1, adult values). PAP 1 activity was localized both in microsomal and cytosolic fractions, the activity in the latter being 4.2 times higher than in the former in adult rats. PAPase activity in microsomal and cytosolic fractions from aged brain showed significant differences with respect to values from adult rats. In microsomal fractions, PAP 2 activity was 23% higher in aged rats. PAP 1 activity in these fractions underwent no changes with respect to values from adult rats. PAP 1 activity in cytosolic fractions, on the other hand, was drastically inhibited (57%) in aged brain, resulting in the inhibition of total PAPase.

Mg^{2+}-dependent and Mg^{2+}-independent forms of the enzyme are described in many tissues (Brindley et al., 1996), and appear to be involved in different metabolic responses (Brindley, 1984; Jamal et al., 1991). When PAPase activity was determined in rat brain microsomal and cytosolic fraction in terms of Mg^{2+}-dependence/independence, Mg^{2+}-dependent PAPase activity (PAP 1) was found in microsomal and cytosolic fractions (54.1 ± 3.2 and 63.8 ± 2.2 nmol × hours $-\ ^{1}\times$ (mg prot.)$^{-1}$), whereas Mg^{2+}-independent activity (PAP 2) was found only in microsomal fractions of rat brain (48.9 ± 3.2 nmol × $hours^{-1}$ × (mg prot.)$^{-1}$). As shown in Fig. 2 Mg^{2+}-independent PAPase activity associated to microsomal fractions was 31% higher in aged rats. The Mg^{2+}-dependent form of microsomal fraction was not significantly affected by aging. However, the same

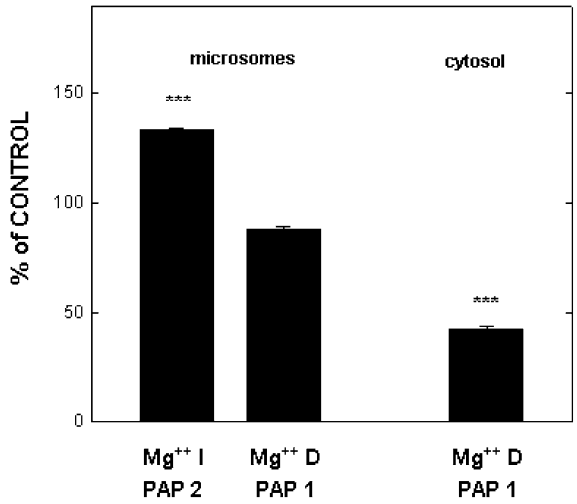

Fig. 2. Effect of aging on metabolization of PA by Mg^{2+}-independent (I) and Mg^{2+}-dependent (D) PAPase activity in microsomal (MIC) and cytosolic (CYT) fractions of brain homogenates. Mg^{2+}-independent activity was determined in the presence of 1 mM EDTA + 1 mM EGTA. Mg^{2+}-dependent activity is expressed as the difference between the activity determined in the presence of 3 mM Mg^{2+} and Mg^{2+}-independent activity. (***$p < 0.001$). Results are expressed as percentage where the control (*adult animals*) is 100%.

form of the enzyme was 64% lower in brain cytosolic fraction from aged rats with respect to adult rats.

Fig. 3 shows that the distribution of the brain PAP 1 form (NEM-S) of PAPase activity between microsomal and cytosolic fractions changes in aged rats with respect to adult rats,

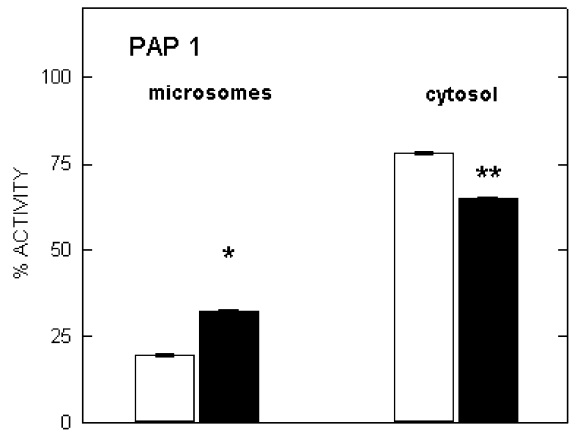

Fig. 3. Effect of aging on the percentual distribution of PAP 1 activity in microsomal and cytosolic fractions from rat brain homogenates. 100% represents the sum of the enzymatic activities determined in microsomal and cytosolic fractions. (*$p < 0.025$; **$p < 0.01$). Adults (□) and aged (■).

Table 1
Effect of aging on monoacylglycerol synthesis in microsomal and cytosolic fractions from brain and liver. MAG synthesis in cytosolic and membrane fractions from both tissues was measured by monitoring the release of [2-^{3}H] monoacylglycerol from of 1,2 diacyl-[2-^{3}H] glycerol (as PAPase product from PAP 1 activity). Enzymatic activities were expressed as DPM of MAG released × hour^{-1} × (mg prot.)$^{-1}$. Each value is the mean ± SD of three individual samples from different animals (three adults and three aged rats). (**$p < 0.01$; ***$p < 0.001$)

	Microsomal fraction		Cytosolic fraction	
	Adult rats	Aged rats	Adult rats	Aged rats
Brain mag	4271 ± 111	8159 ± 632***	5615 ± 408	6743 ± 439
Liver mag	6144 ± 821	8279 ± 184**	5183 ± 112	3120 ± 374

showing an increase in the microsomal fraction and a diminution in the cytosolic fraction of a similar order of magnitude (15%).

MAG production was determined through the metabolization of the product of PAPase activity through NEM assay. As shown in Table 1, there is increased MAG formation from DAG produced by PAPase in aged brain microsomal fraction. A negligible amount of water soluble products such as lysophosphatidic acid and glycerol phosphate was also present (data not shown).

The effect of aging on PAP 2 and PAP 1 activity in liver microsomal and cytosolic

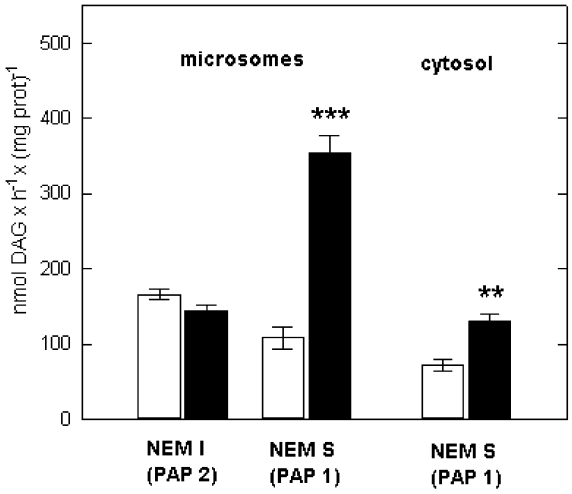

Fig. 4. Metabolization of phosphatidic acid by NEM-S (PAP 1) and NEM-I (PAP 2) phosphatidate phosphohydrolase activities in microsomal (MIC) and cytosolic (CYT) fractions of liver homogenates from adult and aged rats. PAP 1 activity was determined from the total activity measured with Mg^{2+} (3 mM) and NEM (4.2 mM). Parallel incubations were performed after pre-incubating the enzyme with 4.2 mM NEM for 10 min. The difference between these two activities was taken as PAP 1; adults (□) and aged (■). PAP 2 represented PAPase activity measured in the presence of NEM (4.2 mM), without Mg^{2+} (1 mM EDTA plus 1 mM EGTA were carried out with the substrate); adults (□) and aged (■). Enzymatic activities were expressed as nmol of DAG released × hour^{-1} × (mg prot.)$^{-1}$. Each value is the mean ± SD of three individual samples from different animals (three adults and three aged rats). (**$p < 0.01$; ***$p < 0.001$).

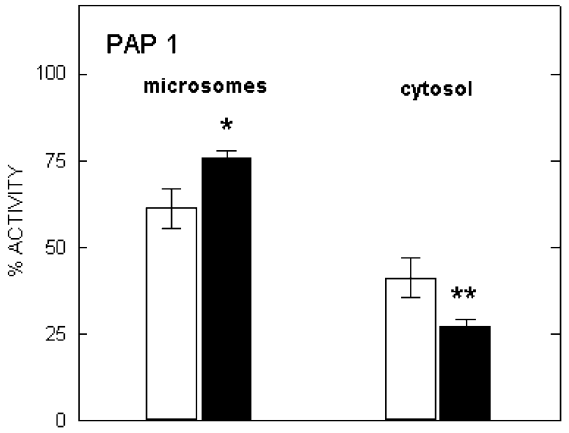

Fig. 5. Effect of aging on the percentual distribution of PAP 1 activity in microsomal (MIC) and cytosolic (CYT) fractions from rat liver homogenates. 100% represents the sum of the enzymatic activities determined in microsomal and cytosolic fractions. (*$p < 0.025$; **$p < 0.01$). Adults (□) and aged (■).

fractions is shown in Fig. 4. PAP 2 activity was only observed in the microsomal fraction in adult rats and was 50% higher than PAP 1 activity for the same fraction (Fig. 4, adult values). PAP 1 activity was localized in microsomal and cytosolic fractions, the activity in microsomal fractions from adult rats being 32% higher than the activity measured in cytosolic fractions. Aging strongly increased PAP 1 activity (230%) with respect to adult rats, whereas PAP 2 activity showed no changes in liver microsomal fractions from aged rats with respect to adult rats. PAP 1 activity measured in cytosolic fractions was stimulated by 73% in liver tissue of aged rats with respect to adults rats (Fig. 4). *Mg^{2+}-dependent/independent activity has not been assessed in the liver*. The effect of aging on the distribution of PAP 1 activity between microsomal and cytosolic fractions from rat liver homogenates is shown in Fig. 5. The percentage distribution of PAP 1 activity was 14% higher in the microsomal fraction and 14% lower in the cytosolic fraction with respect to adult rats, respectively (Fig. 5).

As shown in Table 1, microsomal synthesis of MAG from DAG produced by PAPase activity (PAP 1) was also increased by aging. In addittion, a redistribution of this activity occurs, increasing in the microsomal fraction and decreasing in the cytosolic fraction.

In order to determine whether our results reflect changes that are exclusively due to modifications in enzyme activity or whether they depend more on the membrane composition of fractions obtained at different ages, 5′ nucleotidase and NADPH-cytochrome-c-reductase activity were measured.

Cytosolic fractions showed a negligible level of 5′ nucleotidase. The enzyme activity in microsomal fractions reflected the presence of about 30% of plasma membrane in these fractions. Furthermore, the distribution of 5′ nucleotidase and NADPH-cytochrome c reductase activity in membranes was the same in membrane preparations from adult and aged rats. The ratio of NADPH-cytochrome c reductase activity in microsomal fractions with respect to cytosolic fractions, was 3.8 ± 1 and 4.4 ± 0.5 for adult and aged rats,

respectively. These data indicate that aging does not modify the plasmatic membrane distribution between subcellular fractions.

4. Discussion

Phosphatidate phosphohydrolase plays a key role in glycerolipid synthesis, producing diacylglycerol by dephosphorylating phosphatidic acid. DAG is the precursor of the synthesis of neutral and zwitterionic lipids. In addition, as DAG and PA are potent signaling molecules (Brindley et al., 1996; English et al., 1997; Youwei et al., 1996), PAPase plays an important role in cellular signal transduction and is an ubiquitous enzyme in several tissues (Brindley, 1984; Coleman and Hubscher, 1963; Jamdar and Cao, 1994; Pasquaré de García and Giusto, 1986). Two forms of mammalian PAPase can be identified taking into account subcellular localization and enzyme properties (Jamal et al., 1991). PAP 1, an Mg^{2+}-dependent and NEM sensitive enzyme related to lipid synthesis, exists in both soluble and membrane associated forms. PAP 2, an Mg^{2+}-independent and NEM insensitive enzyme, is mainly, though not exclusively, an integral plasma membrane protein (Fleming and Yeaman, 1995; Jamdar and Cao, 1994) involved in signal transduction mechanisms via the phospholipase D pathway (Walton and Possmayer, 1985). Very recently, the PAP 2 family was proposed to be renamed as lipid phosphate phosphohydrolases (LPPs) since they hydrolize a variety of lipid phosphates (Brindley and Waggoner, 1998).

PAPase activity was measured taking into account Mg^{2+}-ion dependence and different NEM sensitivity of PAP 1 and PAP 2 isoforms. Our results demonstrate that the effect of aging on NEM-sensitive PAPase matches the behaviour of the Mg^{2+}-dependent form of the enzyme and that the effect of aging on NEM-insensitive activity matches the effect on the Mg^{2+}-independent form.

We have found that in brain and liver, PAP 1 is associated both with the microsomal and cytosolic fraction whereas PAP 2 is exclusively associated with the microsomal fraction. Liver microsomal PAP 1 and PAP 2 activity in adult rats was higher than that in brain tissue. On the contrary, liver cytosolic PAP 1 activity observed in adult rats was lower than that observed in adult brain tissue. In brain and liver microsomal fractions from adult rats, the PAP 2 form was higher than the PAP 1 form. In this respect, it has been demonstrated that highly-purified myelin from rat brain stem contains PAP 2 and that it is intrinsic to myelin membranes (Vaswani and Ledeen, 1989).

Our results demonstrate important and quantitatively differential changes produced by aging in PAP 1 and PAP 2 activity in microsomal and cytosolic fractions from brain and liver. Aging decreases total brain PAP 1 (microsomal plus cytosolic) activity. Moreover, when analyzing the effect of aging on brain PAP 1 distribution in microsomal and cytosolic fractions we found the enzyme to be translocated from cytosolic to microsomal fractions. Aging thus provokes no changes in microsomal PAP 1 activity in brain tissue whereas total PAP 1 activity decreases. It is possible that this decrease passes unnoticed as it may be compensated by translocation of the enzyme from cytosolic to microsomal fractions; this could in turn explain the absence of any observed effects in the biosynthesis of PC and PE, measured through the incorporation of [2-^{3}H]-glycerol in brain tisular suspension of aged rats. (Ilincheta de Boschero et al., 2000a).

Total liver PAP 1 activity on the other hand is dramatically increased by aging, microsomal and cytosolic activity increasing by 245 and 82%, respectively. The percentage distribution of PAP 1 activity showed an increase in microsomal fractions with respect to adult values. These results demonstrate that aging, apart from increasing total PAP 1 activity, favors its translocation from cytosolic to microsomal fractions. The cytoplasmic form of PAP 1 acts as a reservoir of activity which moves onto the endoplasmic reticulum to become metabolically active. PAP 1 translocation could be then produced by an accumulation of fatty acids, acyl CoA esters or phosphatidate within the membranes (Balsinde and Dennis, 1996; Brindley, 1984; Butterwith et al., 1984; Cascales et al., 1984; Martin-Sanz et al., 1984, 1985). Other enzymes involved in lipid metabolism are also regulated by translocation. Fatty acids and DAG appear to promote cytidylyltransferase translocation to the endoplasmic reticulum and more CDP-choline is produced for PC synthesis (Brindley, 1984).

It has been reported that the rate of PE and PC synthesis from CDP-bases is lower in brain microsomes from 18-month-old rats than in adult animals and that the exogenous DAG addition to microsomes from aged rat brain increases the rate of synthesis almost to that of adult levels (Brunetti et al., 1979). It has also been suggested that the diminution of unsaturated DAG could be one of the possible reasons for biosynthesis decline. In cerebral cortex, aging does not change microsomal PAP 1 activity whereas the total PAP 1 activity undergoes a decrease. PAP 1 translocation from cytosolic to microsomal fractions in 28.5 month-old rats (Fig. 3) can therefore be interpreted as a mechanism to achieve the appropiate diacylglycerol levels for PC and PE synthesis. However, no variations in the novo PC and PE synthesis (known to be mainly microsomal located) were observed in brain cell suspensions of 28.5 month-old rats (Ilincheta de Boschero et al., 2000a). In addition, we found that MAG production increases in brain microsomal fraction (Table 1). Translocation of PAP 1 could act as a feed-forward regulator for these glycerophospholipid synthesis, but increased DAG metabolization could explain the impairment to subsequent DAG transformations.

Normal aging is accompanied by an age-related decline in insulin sensitivity (Escriva et al., 1997; Ishizuka et al., 1993; Qu et al., 1999) in liver, adipocytes and skeletal muscle. The decrease in insulin sensitivity occurring during normal aging could thus be related with a decrease in glucose uptake, increased lypolitic activity, and increased free fatty acid availability in serum (Higgins et al., 1999). It has also been reported that age induces a reduction in rat liver fatty acid binding protein (FABP) (Singer et al., 1996). The increased availability of fatty acids has also been related with translocation of PAPase from cytosol to microsomes (Cascales et al., 1984).

Changes in liver PAPase activity in diabetes and stress, when altered ratios of glucocorticoids (such as other stress hormones) relative to insulin are present, have been also suggested to be responsible for increased PAPase synthesis in the liver (Lawson et al., 1982). These conditions also cause the mobilization of fatty acids from adipose tissues.

As mentioned previously, a PAPase translocation to membranes by free fatty acids (oleic acids and other long chain fatty acids) has been extensively reported (Cascales et al., 1984; Martin-Sanz et al., 1984). Our results not only revealed increased translocation but also increased PAP 1 microsomal activity in liver as a result of aging. PAP 1 behaviour in liver tissue, different from that in brain, could be related with alterations in hormonal balance.

PAP 2 or PAP 1 activities have been localized preferentially in plasma membrane or cytosol and endoplasmic reticulum, respectively (Jamal et al., 1991). The determination of the plasma membrane marker enzyme 5′ nucleotidase activity in cytosolic fraction showed a negligible proportion to be present in the cytosolic fraction. In the present study, the microsomal fraction was often found to contain plasma membrane (as much as 30%, as evidenced by the presence of 5′ nucleotidase activity). Therefore, it is likely that PAP 2 associated with the microsomal fraction may be from plasma membrane. It is interesting to note that aging does not induce modifications in the distribution of marker enzymes, implying that aging-induced changes in PAPase activity are not a consequence of modified membrane distribution.

In accordance with other authors (Jamdar and Cao, 1994), we have found substantial PAP 2 activity in microsomal fractions from brain and liver tissue. Microsomal PAP 2 form in rat brain is stimulated by aging, whereas the same form in liver microsomal fractions of aged rats shows no changes with respect to adults. As PAP 2 enzyme is an integral membrane protein always present in an active state, it is likely that this form of the enzyme is involved both in the metabolism of membrane phospholipids and in the signal transduction mechanisms mediated by phospholipase D (PLD), as previously proposed (Jamal et al., 1991; Martin et al., 1993) for other tissues. In this connection we recently reported increased PLD activity in cerebral cortex homogenates from 28.5-month-old rats (Ilincheta de Boschero et al., 2000b).

A novel aspect of the function of PAP 2 has been recently disclosed by its broad substrate specificity. It has been demonstrated that Lyso PA, ceramide 1-Phosphate and sphingosine 1-Phosphate are substrates of this enzyme (Hooks et al., 1998; Kai et al., 1997; Roberts et al., 1998). Given that ceramide, sphingosine, and their phosphorylated derivatives are known to serve as signaling molecules (Hannun, 1994; Kolesnick and Golde, 1994; Spiegel et al., 1996), PAP 2 activity can be a key regulatory enzyme in the metabolic processing of lipid mediators derived from glycerolipids and sphyngolipids. The PAP 2 form from rat liver has been described as being capable of hydrolyzing DAG pyrophosphate (Munni et al., 1996), a novel phospholipid with a potential signaling function. In addition, PAP 2 isozymes termed PAP 2a, PAP 2b and PAP 2c have been recently described (Hooks et al., 1998; Kai et al., 1997; Roberts et al., 1998). Whereas PAP 2a has been shown to have the ability to degrade all mentioned phosphorylated intermediates, PAP 2b was only able to hydrolyze sphingosine 1-phosphate. Increased PAP 2 activity in membranes could also have interesting physiological implications in terms of a higher rate of removal and appearance of PA and DAG, metabolic intermediates involved in signal transduction. We have measured PAP 1 and PAP 2 activities employing only PA as substrate; further studies will be necessary to determine the behaviour of the different PAP 2 isoforms in aged brain.

Previous findings in retina (Rotstein et al., 1987) and brain from aged rats (unpublished observations) lead us to suggest that the neural structure of aged animals attempts to counteract the changes in lipid composition through a more efficient utilization of docosahexaenoic acid as well as other unsaturated fatty acids (López et al., 1995). The increased translocation of brain and liver PAP 1 from cytosol to microsomes in aged rats also indicates a selective compensatory mechanism in membranes for the regulation of glycerolipid synthesis. The balance between negative effects and compensatory

mechanisms cannot counteract the effects of aging, and significant modifications in membrane composition are therefore observed (López et al., 1995). These preliminary observations provide us with a starting point to evaluate the effect of aging on glycerolipid synthesis in the brain and liver. It has been recently suggested that a logical explanation for the different subcellular location of PAPase activities is that they provide independent pools of DAG for specific cellular functions (Vance, 1998). The differential behaviour of different types of PAPases, their isoforms and their distribution during aging, open up complex avenues for further study.

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